Elafin (elastase-specific inhibitor) has anti-microbial activity against Gram-positive and Gram-negative respiratory pathogens

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Abstract Elafin (elastase-specific inhibitor) is a low molecular weight inhibitor of neutrophil elastase which is secreted in the lung. Using synthetic peptides corresponding to full-length elafin (H_2N-^{1}AVT.....^{95}Q-OH), the NH_2-terminal domain (H_2N-^{1}AVT.....^{50}K-OH) and the COOH-terminal domain (H_2N-^{51}PGS.....^{95}Q-OH), we demonstrate that elafin's anti-elastase activity resides exclusively in the COOH-terminus. Several characteristics of elafin suggest potential anti-microbial activity. The anti-microbial activity of elafin, and of its two structural domains, was tested against the respiratory pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Elafin killed both bacteria efficiently, with 93% killing of *P. aeruginosa* by 2.5 μM elafin and 48% killing of *S. aureus* by 25 μM elafin. For both organisms, full-length elafin was required to optimise bacterial killing. These findings represent the first demonstration of co-existent anti-proteolytic and anti-microbial functions for elafin.

Key words: Elafin; Human neutrophil elastase; Anti-microbial activity; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

As part of the immediate defence against bacterial pathogens, the lung is equipped with a number of endogenous anti-microbial peptides, including defensins, secretory leucocyte protease inhibitor (SLPI), lysozyme and lactoferrin [1–6]. In addition, bacteria stimulate the recruitment and activation of neutrophils, resulting in engulfment of organisms and ultimately their destruction by oxidants or proteolytic enzymes such as human neutrophil elastase (HNE) [7]. Natural inhibitors of HNE in the lung are thought to play a critical role in neutralising HNE liberated extracellularly, thus circumventing proteolytic damage to the host [8].

Elafin (also known as elafin-specific inhibitor (ESI) or skin anti-leucoprotease (SKALP)) is a low molecular weight (9.9 kDa) inhibitor of HNE and proteinase 3, which is secreted in the respiratory tract [9,10]. Along with α_1-protease inhibitor (α_1-PI) [8] and SLPI [11], elafin comprises an integral part of the 'anti-elastase shield' in the lung [12].

Several features suggest an additional anti-microbial function for elafin. In particular, elafin is highly cationic, it is expressed selectively in the lung, the skin and at mucosal surfaces and SLPI (which has 42% sequence homology with elafin) has anti-microbial activity [5,13–17].

In this study, we describe an investigation of the anti-microbial activity of elafin against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two important pulmonary pathogens with a particular propensity to antibiotic resistance [18–23].

2. Materials and methods

2.1. Proteins

Elafin peptides were produced synthetically in accordance with the derivation of amino acids from the established gene sequence [13] and were supplied by Albachem (Edinburgh, UK), using standardised protocols described elsewhere [24]. Three peptides were provided, namely full-length elafin (H_2N-^{1}AVT.....^{95}Q-OH), the NH_2-terminus domain (H_2N-^{1}AVT.....^{50}K-OH) and the COOH-terminus domain (H_2N-^{51}PGS.....^{95}Q-OH). The terminal amino acid of the NH_2 domain and the first amino acid of the COOH domain were determined by analysis of the established crystal structure of a 57 amino acid fragment of elafin (H_2N-^{39}AQE.....^{95}Q-OH) [17], in conjunction with the established sequence of human SLPI, which has 42% sequence homology with elafin [13–15,25]. The molecular weights of elafin moieties, determined by mass spectrometry (Albachem, Edinburgh, UK) were 9925 Da for full-length elafin, 5172 Da for the NH_2-terminal domain and 4776 Da for the COOH-terminal domain.

Human serum albumin (HSA) was purchased from Sigma Chemicals (St. Louis, MO, USA). Recombinant human SLPI was purchased from R and D Systems (Minneapolis, MN, USA). Lyophilised preparations of elafin, SLPI and HSA were all reconstituted in 0.01 M K_2HPO_4/KH_2PO_4, pH 7.4 (phosphate buffer).

2.2. Bacteria

PAO1, a clinical strain and well-characterised type strain of *P. aeruginosa*, and C1705, a clinical strain of *S. aureus*, were available in-house [20,24].

2.3. HNE activity assay

The HNE activity assay has been described in detail elsewhere [26]. Briefly, serial dilutions of test inhibitor were added to 300 ng of purified HNE (Elastin Products, Owensville, MO, USA). All dilutions were performed in Tris 50 mM, Triton 0.1%, sodium chloride 0.5 M, pH 8.0. The samples were incubated for 15 min at 37°C before addition of the chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma). In positive controls, buffer replaced test inhibitor. The change in absorbance, measured spectrophotometrically at 405 nm (MR5000 Plate Reader, Dynatech, Dynex, Billinghurst, UK), was expressed as a function of time. The inhibitory capacity of test inhibitors was derived by extrapolation to the ordinate of curves obtained by plotting the HNE activity against the concentration of inhibitor added, as described elsewhere [27].

2.4. Assay of microbial activity

Bacteria were grown initially as colonies on Columbia agar (Unipath, Basingstoke, UK) and, then, in 10 ml tryptone soya broth (TSB) (Unipath) overnight at 37°C in an orbital shaker (Gallenkamp, Fisher Scientific, Loughborough, UK) at 200 rev/min. 100 μl of the bacterial culture was resuspended in 10 ml fresh TSB and incubated for 3 h at 37°C with rotation, corresponding to a point compatible with logarithmic growth for both PAO1 and C1705. The resulting suspension

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Abbreviations: HNE, human neutrophil elastase; α_1-PI, α_1-protease inhibitor; SLPI, secretory leucocyte protease inhibitor; HSA, human serum albumin; TSB, tryptone soya broth; LPS, lipopolysaccharide; CF, cystic fibrosis

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was centrifuged at 4500 rpm for 20 min at room temperature (Biofuge, Heraeus Instruments, Kendro, Bishops Stortford, UK), the supernatant discarded and replaced with 10 ml phosphate buffer and then resuspended. This process was repeated once. The quantity of viable bacteria was calculated from pre-constructed growth curves and dilutions made in phosphate buffer to give an estimated count of 5×10^4 viable colonies per ml.

In experiments using purified elafin, HSA or SLPI, 30 μl aliquots of the bacterial suspension were added to 90 μl aliquots of test substance, each diluted in phosphate buffer to give final concentrations of 1, 2.5, 10 or 25 μM of the test substance. In positive controls, 90 μl of phosphate buffer replaced the test solution and in negative controls, 30 μl of phosphate buffer replaced the bacterial suspension.

The test bacterial suspension mix was incubated for 2 h at 37°C. Appropriate dilutions were made in phosphate buffer and 100 μl aliquots plated out on Columbia agar. Colonies were counted after incubation of the plates for 16 h at 37°C.

2.5. Statistics

Data pertaining to *S. aureus* were normally distributed and comparisons between test substances and controls were performed using the paired *t*-test. Data pertaining to *P. aeruginosa* were not normally distributed and comparisons between test substances and controls were performed using the Wilcoxon signed rank test. Statistical significance was regarded as $P < 0.05$.

3. Results

3.1. Anti-elastase activity of elafin moieties

All three peptides (full-length elafin, the NH_2-terminal domain and the COOH-terminal domain) were tested for anti-HNE activity (Fig. 1). No anti-HNE activity was detected in the NH_2-terminal domain. In contrast, the COOH-terminal domain and the full-length molecule were found to have an identical anti-HNE activity, indicating that elafin's anti-elastase activity resides exclusively in the COOH-terminal domain.

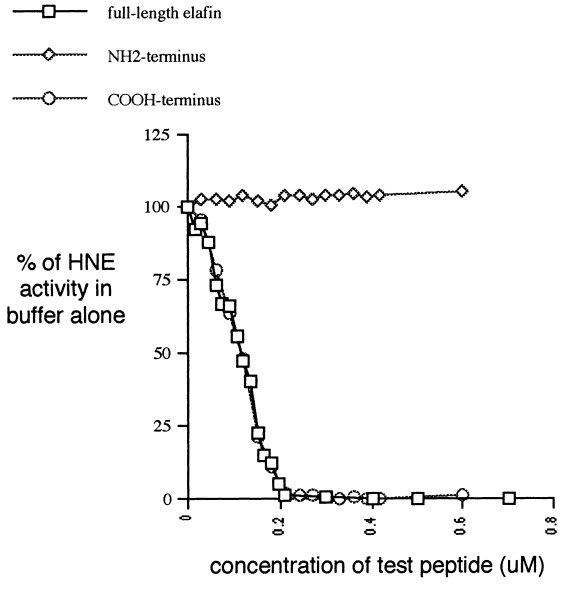

Fig. 1. Anti-elastase activity of full-length elafin, NH_2-terminal domain elafin and COOH-terminal domain elafin. Known quantities of test inhibitor were added to 300 ng HNE. All dilutions were performed in Tris 50 mM, Triton 0.1%, sodium chloride 0.5 M, pH 8.0. Chromogenic substrate (*N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide) was added and the change in absorbance at 405 nm was measured as a function of time. The molecular weights of full-length elafin, NH_2-terminal domain elafin, COOH-terminal domain elafin and HNE are 9.9, 5.2, 4.8 and 30 kDa, respectively. Results are expressed as a percentage of the HNE activity when incubated with buffer alone.

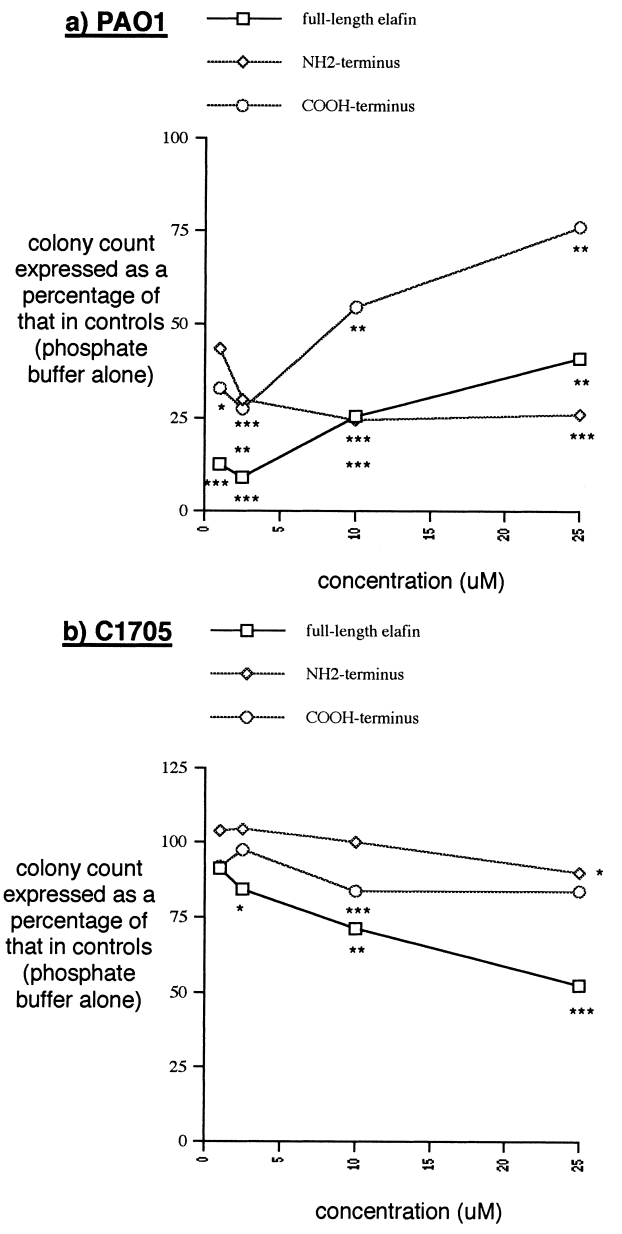

Fig. 2. Anti-microbial effect of elafin moieties against *P. aeruginosa* (PAO1) and *S. aureus* (C1705), expressed as a percentage of the colony count in phosphate buffer alone (control). In (a), results represent medians ($n = 8$ for concentrations of 2.5–25 μM; $n = 4$ at 1 μM). In (b), results represent means ($n = 5$). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

3.2. Anti-microbial activity of elafin

Full-length elafin resulted in a significant killing of PAO1 at all doses tested (1–25 μM) (Fig. 2). The maximum effect was observed at 2.5 μM, at which 93% of PAO1 was killed, relative to PAO1 grown in phosphate buffer alone. At 1 and 2.5 μM, the contributions of the NH_2-terminus and COOH-terminus were approximately additive, but at higher doses, the predominant anti-microbial effect resided in the NH_2-terminal domain. The anti-microbial effect of full-length elafin against

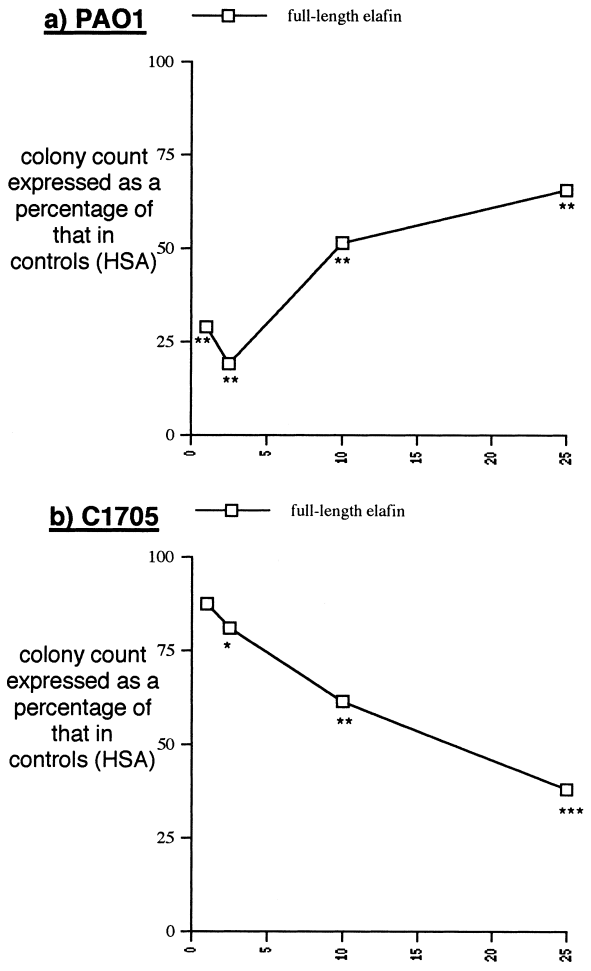

Fig. 3. Anti-microbial effect of full-length elafin against *P. aeruginosa* (PAO1) and *S. aureus* (C1705), expressed as a percentage of the colony count in HSA. In (a), results represent medians ($n=8$ for concentrations of 2.5–25 μM; $n=4$ at 1 μM). In (b), results represent means ($n=5$). Comparisons were made between equimolar concentrations of test peptide and HSA. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. For reasons of visual clarity, only data for full-length elafin are shown, however, dose-response curves for NH_2-terminal domain elafin and COOH-terminal domain elafin also closely paralleled those shown in Fig. 2.

S. aureus (C1705) was dose-dependent and was significant over a dose range of 2.5–25 μM (Fig. 2). At 25 μM, 48% killing of C1705 was achieved. The NH_2-terminus showed a significant anti-microbial activity only at 25 μM and the COOH-terminus only at 10 μM. To ensure that the effects observed were not attributable to a non-specific peptide effect, the effects of full-length elafin, the NH_2-terminal domain and the COOH-terminal domain against PAO1 and C1705 were also compared with a control protein (HSA) (Fig. 3). For full-length elafin, the maximal activity was described at 2.5 μM against PAO1 (81% killing) and at 25 μM against C1705 (63% killing) (Fig. 3), closely in keeping with the findings when the effect of full-length elafin was compared with that of phosphate buffer alone (Fig. 2). The dose-response curves generated for both NH_2-terminal domain elafin and COOH-terminal domain elafin against PAO1 and C1705 also closely paralleled those obtained when survival was expressed relative to PO_4 buffer instead of HSA (data not shown).

3.3. Anti-microbial activity of SLPI

In a separate set of experiments, the activity of SLPI against PAO1 and C1705 was tested over the dose range 1–10 μM and compared with that of full-length elafin (Table 1). The anti-microbial activity of elafin against PAO1 conformed to the characteristic pattern shown in Fig. 2, with a maximal effect at 2.5 μM. The anti-microbial activity of elafin was greater than that of SLPI at all doses tested. To test for potential synergy, equimolar concentrations of SLPI and elafin (0.5 μM elafin added to 0.5 μM SLPI) were compared with either 1 μM elafin or 1 μM SLPI alone. No evidence of a synergistic effect was found (86% killing of PAO1 using the elafin/SLPI combination, 88% using elafin alone, 80% using SLPI alone). The anti-microbial activity of elafin and SLPI against C1705 was similar over the dose range tested.

4. Discussion

Elafin (ESI/SKALP) was originally characterised and sequenced from human bronchial secretions and from human psoriatic skin, on the basis of its anti-elastase activity [9,14]. The identification of anti-microbial activity against Gram-negative and Gram-positive respiratory pathogens at concentrations potentially achievable in epithelial lining fluid [28] (especially after genetic augmentation [26]) suggests more complex functions for elafin in the context of inflammation. This observation is in keeping with the identification of additional functions for other major anti-elastases such as SLPI, which has intrinsic anti-bacterial and anti-viral activity [5,16,29] and which can influence the function of lipopolysaccharide (LPS) [30] and prostaglandins [31].

Indeed, elafin can be added to the growing list of endogenous lung peptides which harbour an anti-microbial function, including defensins, SLPI, lactoferrin and lysozyme [1–6]. These molecules share certain general characteristics, for example, a low molecular weight and net positive charge. However, each has unique structural features, which may play a role in determining the anti-microbial function. The gene sequence and derived amino acid sequence of elafin have allowed identification of various structural determinants [13]. The NH_2-terminal domain, as defined in this study, has a net positive charge of +5, as well as repeated structural motifs potentially acting as substrate for transglutaminase which may allow elafin to bind to the interstitium covalently [13,15,32]. The COOH-terminal domain, as defined in this study, has a net positive charge of +2 and has four disulfide bonds which confer structural stability [13,32]. Previous studies using elafin fragments of either 57 amino acids (H_2N-

Table 1
Anti-microbial effect of full-length elafin and SLPI against *P. aeruginosa* (PAO1) and *S. aureus* (C1705)

PAO1	1 μM	2.5 μM	10 μM
Full-length elafin	95.7	96.3	85.1
SLPI	93.8	94.7	73.1
C1705			
Full-length elafin	−9.8	−7.9	13.6
SLPI	−8.1	18.1	6.0

Results are expressed as percentage killing relative to that in PO_4 buffer alone (taken as 0%) and represent medians from three separate experiments.
When expressed relative to HSA, the results obtained were similar and followed the same trend (data not shown).

^{39}AQE.....^{95}Q-OH) or 48 amino acids (H_2N-^{48}STK.....^{95}Q-OH) have demonstrated high affinity anti-elastase activity (K_i in the nM range), but were unable to provide information concerning the NH_2-terminal end of the molecule [9,14,33]. These findings, in conjunction with crystallographic studies [17], suggested that elafin's anti-elastase activity resides exclusively in the COOH-terminal domain. Using discrete functional domains of elafin and studying full-length elafin for the first time, we have been able to provide direct confirmatory evidence to support this hypothesis by demonstrating that the COOH-terminal domain of elafin and the full-length molecule bind HNE with an equal efficiency (Fig. 1).

Furthermore, the use of discrete functional domains of elafin has allowed us to infer which regions of the molecule may be responsible for anti-microbial effects. In the case of *P. aeruginosa*, our data suggest that inherent anti-microbial activity exists in both structural domains and imply that their interaction may be necessary to augment anti-microbial activity at low concentrations. The inverse dose-response relationship noted may reflect the ability of *P. aeruginosa* to use higher concentrations of peptide as nutrients, promoting growth and competing with the killing effect of elafin. Indeed, *P. aeruginosa* is known to grow in increasing concentrations of organic nitrogen [34]. This may explain why elafin's anti-microbial effect was slightly less pronounced when albumin was used as control (see Fig. 3).

The anti-microbial effects of several low molecular weight peptides have been ascribed to their cationic nature. Cationic peptides are capable of binding *P. aeruginosa* LPS, altering the outer membrane stability and increasing susceptibility to other bactericidal compounds [35]. While the charge may have contributed to the killing observed here, it is unlikely to explain the entire anti-microbial effect of elafin (especially at low doses, where the less cationic COOH-terminal domain was more effective).

The anti-microbial activity against *P. aeruginosa* was independent of the anti-elastase activity of elafin, as the majority of anti-microbial activity resided in the NH_2-terminal domain, while anti-elastase activity resided exclusively in the COOH-terminal domain. Interestingly, a quite different, dose-dependent pattern of anti-microbial activity was seen against *S. aureus*. The NH_2-terminal domain alone contributed only slightly and at high concentrations. As with *P. aeruginosa*, this suggests that a simple charge effect is unlikely to explain elafin's anti-microbial effect. The observation that full-length elafin was more effective than the additive effects of the two structural domains may again imply a critical interaction between these. In a similar study using structural domains derived from SLPI, Hiemstra et al. [5] also found that the full-length molecule was more active against *Escherichia coli* and *S. aureus* than was either terminal fragment.

In our hands, SLPI was found to have anti-microbial activity against *P. aeruginosa* and *S. aureus* as has been described elsewhere [5,16]. The anti-microbial effects of SLPI were less pronounced in our study, but this may reflect the use of different clinical strains. Our data suggest that in equimolar concentrations, elafin is at least as effective as SLPI against PAO1.

The anti-microbial activity of elafin adds to the emerging picture of its primary role in the lung defence. Elafin is ideally placed to promote the early eradication of invading pathogens and to protect the host against proteolytic destruction in the event of neutrophil recruitment. Indeed, the co-existence of anti-microbial and anti-elastase activity in the elafin molecule could have therapeutic implications. *P. aeruginosa* and *S. aureus* can each cause severe pneumonia [18,21] and frequently co-colonise patients with cystic fibrosis (CF) [19]. The significant morbidity and mortality associated with these organisms, coupled with their propensity to develop resistance to conventional antibiotics [22,23], demands the development of novel anti-microbial strategies. In CF, HNE is thought to contribute to the airway pathology by degrading substrate in the interstitium, enhancing inflammatory cell chemotaxis, stimulating mucous hypersecretion and promoting the adherence of *P. aeruginosa* [36–38]. Furthermore, PAO1 is known to promote significant release of elastase from hamster neutrophils in vivo [39]. Effective augmentation of anti-microbial anti-elastases for patients known to be at risk of developing infection with *P. aeruginosa* and/or *S. aureus* may thus be theoretically desirable. Our findings suggest that elafin gene augmentation could be particularly beneficial in CF. Elafin levels are known to be reduced in CF [40], high levels of elafin can be effected using adenoviral gene therapy in rats in vivo [26], elafin's transglutamination sites may confer a longer biological half-life in vivo [15,32] and elafin appears particularly active against the non-mucoid clinical isolate PAO1. Strategically, eradication of *P. aeruginosa* whilst still in the non-mucoid form may be especially important in preventing or delaying progression to chronic infection with mucoid variants, which are seldom eradicated and are associated with a significantly worse prognosis in CF [20].

We recently showed that genetic augmentation of elafin using adenoviral gene therapy protects human alveolar epithelial (A549) cells against HNE and activated neutrophils [41]. We have extended these findings to show that elafin's anti-microbial activity against PAO1 can be augmented in supernatant derived from A549 cells transfected with adenovirus encoding elafin (unpublished data). This suggests that genetic augmentation of endogenous anti-microbials may be effective against pulmonary pathogens, as has also been demonstrated using γ-interferon [42,43].

In summary, these findings demonstrate for the first time that elafin has an intrinsic anti-microbial activity against important Gram-negative and Gram-positive respiratory pathogens and that this activity is independent of the molecule's anti-elastase activity.

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